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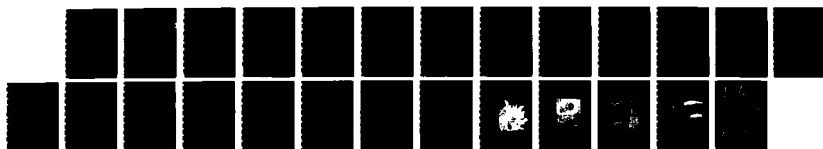
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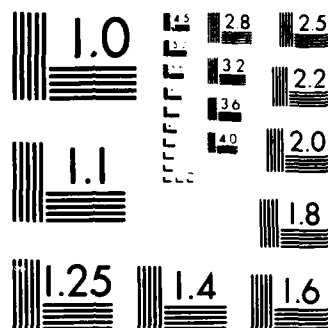
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Caulobacters in the Marine Environment. Smit, John

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CAULOBACTERS IN THE MARINE ENVIRONMENT

John Smit

SUMMARY

Marine Caulobacters are bacteria that elaborate a cellular stalk and attach to surfaces via an adhesion organelle (the holdfast) at the tip of the stalk. We have begun a study which focuses on this group of bacteria as an example of microbial biofouling and describe the isolation and preliminary characterization of new strains of marine Caulobacters. Caulobacters could be isolated from all marine sampling locations. Strains were distinguishable by a variety of morphological and biochemical criteria. All required at least some percentage of seawater in the growth medium. About 60% of the isolates could grow anaerobically. Preliminary analysis of the holdfast of numerous isolates showed that all bound the lectin wheat germ agglutinin in preference to a group of other lectins, indicating the presence of N-acetylglucosamine. In contrast, a group of freshwater Caulobacters showed more diversity in holdfast composition, based on analysis with a panel of lectins. In all Caulobacters tested, the holdfast also bound to particles of colloidal gold and silver. Initial work in the development of genetic cloning capabilities of marine Caulobacters is also presented, with the longterm goals of using this technology in the analysis of holdfast composition and regulation, the study of other factors involved with survival in a biofilm and the development of beneficial uses for this group of marine bacteria.

INTRODUCTION

It is widely understood that biofouling marine bacteria are the first type of organism to populate a surface placed in the ocean, thereby comprising the first stage of a progression that leads to a complex macrofouling community. But relatively little is known about the adhesive substances produced by these bacteria (Sutherland, 1983), the range of mechanisms used to attach and adhere to surfaces or the dynamics involved with survival in biofilms.

Marine Caulobacters represent one distinctive type of biofouling bacteria, those that

attach using an adhesive organelle located at a discrete location on the cell surface(Poindexter, 1964). I have chosen to study the factors involved with biofouling by marine Caulobacters, partly because the discrete location and function of the holdfast allows development of simpler, more unambiguous methods to analyze and isolate the adhesive substance than is possible for many other marine biofouling bacteria. Also, it is expected that past experience with the biochemistry, physiology and genetics of freshwater Caulobacters will accelerate work with the marine counterparts(for reviews see Poindexter, 1981a and Shapiro, 1985). Finally, it is expected that the knowledge gained in studying marine Caulobacters as an example of microbial fouling may lead to development of productive uses of these organisms in the marine environment.

This paper describes initial efforts in the isolation of new strains of marine Caulobacters, a preliminary analysis of holdfast composition and the first steps in developing and characterizing the capabilities for doing molecular genetic experiments with these bacteria.

METHODS

Bacterial strains and growth conditions

Isolation of new marine and freshwater Caulobacter strains was accomplished in the following manner. Seawater or freshwater samples were supplemented with peptone to 0.01% and left at room temperature for one to two months. When light microscopy examination of the surface film indicated an adequate number of stalked bacteria were present, loopfuls of the surface film were diluted appropriately, vortexed vigorously and plated onto solid medium. Media contained 2 g peptone, 1 g yeast extract, and 15 g agar per liter of fresh water (PYE) or seawater (S-PYE). Following incubation at 30°C, bacterial colonies were examined by light microscopy. Those containing bacteria with appropriate characteristics(motile cells, stalked cells, attachment to surfaces at the tip of the stalk and formation of rosettes-groups of stalked cells held together at the ends of their stalks) were directly inoculated into S-PYE or PYE liquid medium.

Following growth, these cultures were frozen at -70°C after addition of DMSO to 10%. The strains were subsequently examined for purity by standard methods and only those frozen cultures which contained contaminating bacteria were replaced by a purified culture.

Some of the marine *Caulobacters* used in experiments described below (CM 260 and CM 243) were obtained from Dr. Jeanne Poindexter.

Ability to grow under anaerobic conditions was tested using the Gas-Pack (BBL-Bectin Dickison) system, with the obligate anaerobe *Clostridium sporogenes* used as a control for anaerobic conditions.

Holdfast Analysis

Strains were examined with a panel of 7 fluorescein isothiocyanate (FITC)-conjugated lectins (Vector Laboratories) by adding 2-3 μl of conjugated lectin to 200 μl of culture. After 20 min the mixture was diluted to 1.5 ml with water or seawater and centrifuged. The pellet was suspended in a solution of 50% glycerol, water or seawater and 2% *N*-propyl gallate (which retards photobleaching of fluorescein [Giloh and Sedat, 1982]). The preparations were examined by vertical fluorescence microscopy.

The effect of several glycolytic enzymes on holdfast integrity was evaluated by incubation of enzyme with 200 μl of cells in culture medium or pH-buffered solutions, as required by the particular enzyme. After one to two hr an FITC-conjugated lectin known to bind to the holdfast under examination was added and the mixture processed as above.

The effect of lectins on the adhesion of cells to glass was evaluated for some strains. Cells were treated with lectin, applied to a glass cover slip and after 5 min unbound cells were removed by vigorous application of medium with a squirt bottle. The cover slip was placed on a Petroff-Hausser counting device and attached cells were enumerated.

Electron microscopy

Negative stain or unstained whole mount microscopy was done by standard procedures. For marine strains it was necessary to fix cells with glutaraldehyde before exposing them to stains prepared in distilled water.

Colloidal gold and colloidal silver particles were prepared according to published methods(De Mey, 1983, Smit and Todd, 1986 and Roth, 1982) and were used unconjugated or conjugated with protein A. Cells were exposed to the particles and unbound particles were removed by two cycles of centrifugation and suspension in water.

Molecular genetic methods

Conjugal transfer of broad host-range plasmids from E. coli to marine Caulobacter strains was attempted using standard methods(Willems, 1984), including diparental and triparental matings. Selection against donor E. coli and for recipient Caulobacters was accomplished using rifampicin-containing media and spontaneous rifampicin-resistant mutants of the Caulobacters. Plasmid transfer was confirmed either by testing for resistance to a second drug marker on the plasmid or colony hybridization with radioactive plasmid probes. Radioactive gene hybridization probes were prepared by nick translation procedures(Maniatis et al, 1983). Colony hybridizations were done using standard methods(Maniatis et al, 1983).

RESULTS

Isolation and characterization of Caulobacters

Seawater samples were taken from numerous locations in the Washington State Puget Sound region. Samples were also collected from the San Francisco Bay and waters near Santa Barbara, California. Caulobacters as well as the morphologically similar Hyphomonas were found in virtually all samples. This includes samples taken near sewage outflow, where a wide variety of bacteria were noted after addition of the peptone. About 25 distinct strains of marine Caulobacters have been isolated thus far. If Caulobacters were seen by light microscopy in surface films, it was always possible

to isolate a *Caulobacter* on solid medium, suggesting that most strains will grow on solid media. This was not the case with *Hyphomonas* strains. In most cases where they were noted in surface films, none could be recovered on solid media and those recovered often grew poorly.

None of the marine *Caulobacters* isolated so far could grow without at least some seawater salts in the media. Lower limits have not been established. Sodium chloride could not be substituted for seawater salts. None of the marine *Caulobacters* could use glucose or maltose as sole carbon source. All strains grew at 30°C.

The *Caulobacter* strains isolated were distinguishable in a variety of ways. Although most strains were readily recognized by light microscopy there was considerable variety in such morphological characteristics as stalk length, cell shape, the size of the rosettes formed in culture (Fig 1) and the degree of motility. Stability of the cell in the absence of seawater salts was also variable. If cells were not fixed before negative stain electron microscopy some strains would disintegrate when exposed to distilled water while the appearance of other strains was only slightly affected. There was also wide variation in growth rates in S-PYE medium and in their requirements for oxygen (see below). Polyacrylamide gel electrophoresis of solubilized whole cells showed that nearly all isolates have distinctive protein profiles (not shown). The degree of competency for conjugal transfer of plasmids also was variable (see below).

About 60% of the marine strains grew in anaerobic conditions. A similar percentage was noted when freshwater *Caulobacter* strains (both freshly isolated and those that have been in culture collections for many years) were tested. Growth rates in the anaerobic environment were comparable to aerobic conditions and no striking differences in morphology were noted. These findings are in contrast with the published description of the genus *Caulobacter*, which characterizes the group as obligate aerobes (Holt, 1977).

Preliminary characterization of the holdfast organelle

The results of the lectin binding assay are shown in Tables 1 and 2 and in Fig 2. For the marine *Caulobacters*, the holdfast of virtually all isolates bound wheat germ

agglutinin(WGA) exclusively. The only exception was strain CM 260 which also did not adhere to glass microscope slides and produced no rosettes in culture. It is likely the strain no longer produces a holdfast organelle, probably a consequence of maintenance in laboratory culture.

For several marine strains, N-acetylglucosamine(the monomer sugar that interacts with WGA) was added to the culture at a concentration of 50 mM prior to addition of WGA. No inhibition of binding to the holdfast could be detected.

The freshwater *Caulobacters* showed more variability in the lectin binding assay. Although many also bound WGA, some either did not respond to any of the lectins tested or bound one or more other lectins. All the freshwater strains examined produced a holdfast organelle.

As an additional test of the specificity of WGA binding to holdfast material, the effect of WGA on the ability of MCS18 to attach to glass was assessed (Fig 3). Adhesion was reduced to 3% of the level achieved when a non-binding lectin(*Dolichos biflorus*) was used.

C. crescentus CB2 and MCS 18 were chosen as representative freshwater and marine strains, respectively, to assess the effect of several glycolytic enzymes on holdfast integrity. The FITC-WGA binding assay provided a sensitive measure of the integrity of the holdfast material, especially at the center of rosettes. The enzymes tested thus far are alpha-galactosidase, B-glucosidase, alpha-glucosidase, N-acetyl-glucosaminadase, neuraminidase, and chitinase. Only chitinase had any effect on holdfast material and it only affected the freshwater strain. In that case most rosettes were dissociated and much of the fluorescent-stained material was dissociated from the cells. But the material was not completely disintegrated, indicating that chitinase did not completely degrade the holdfast material.

It was discovered that colloidal gold and colloidal silver particles (both are used as electron microscopy immunocytochemical labels) bind to the holdfasts of marine and freshwater strains (Fig 4). Binding occurred with particles that were coated with Protein A and particles that were stabilized only by polyethylene glycol. There does

not appear to be a correlation with WGA binding. Holdfasts from freshwater strains that do not bind WGA also bound colloidal gold particles.

Preliminary molecular genetic studies.

Marine *Caulobacters* were sensitive to all standard antibiotics used for cloning studies. Moreover, plasmid encoded genes specifying resistance to the antibiotics were expressed in marine strains. Use of tetracycline resistance markers was avoided since seawater compromises the effectiveness of tetracycline. Spontaneous mutants resistant to antibiotics (eg rifampicin) were also readily obtained without mutagenesis.

Attempts to conjugally transfer plasmids from the four major incompatibility groups to the marine strains are underway; in-progress results are shown in Table 3. Representatives from the four major broad host-range incompatibility groups have been successfully introduced into at least one marine *Caulobacter* strain. Some strains are significantly better recipients for conjugation with *E. coli* than others.

Colony hybridization methods were used as a preliminary assessment of the similarity between selected freshwater *Caulobacter* genes and the genomes of marine *Caulobacters*. Probes prepared from the cloned genes for for *Caulobacter crescentus* CB15 flagellins (Milhausen et al, 1982) and the major surface protein (Smit and Agabian, 1984) were used. Both probes faithfully hybridized to all the marine *Caulobacter* isolates while absence of hybridization was noted for a similar sized group of randomly selected marine bacteria (not shown). The homology between the gene probes and the marine *Caulobacters* was not exact; to obtain hybridization it was necessary to choose stringency conditions that permit as much as 25-30% mismatch of base pairs.

DISCUSSION

To initiate a longterm study of biofouling *Caulobacters*, it was necessary to accumulate new isolates whose exposure to laboratory culture conditions has been minimized as much as possible. For example, strains of *Caulobacter crescentus* that have been

cultured for many years in the laboratory for cell development studies, produce little or no holdfast material. Additionally, a crystalline protein surface layer (Smit et al, 1981), which is thought to somehow be a defense against environmental stresses (Smit, 1986), is present on some laboratory strains but absent in others (Smit and Agabian, 1984). Some strains have been shown to have apparently inactivated surface structure genes. The isolation and preservation methods used here are expected to minimize the loss of characteristics not selected for in laboratory conditions.

No quantitative studies were done to estimate the relative abundance of marine *Caulobacters* in the ocean. We did observe however that marine *Caulobacters* could be found in all our samples of seawater. Although they did not seem to ever be the most prevalent genus of marine bacteria, their ubiquitousness was notable.

These initial studies suggest that the various strains of marine *Caulobacters* all possess a holdfast of similar and possibly identical composition. This similarity is in spite of the wide variety of other morphological, physiological, and biochemical characteristics noted for these strains.

In contrast, the freshwater *Caulobacters* showed more variation in holdfast composition. Although WGA binding was still the most common characteristic, a high percentage of strains show other lectin-binding characteristics. One factor for this variability could be that many of the freshwater strains examined have been in laboratory culture for many years, allowing for changes in holdfast composition to occur. But several new isolates also failed to bind WGA. It is possible that when compared to the marine environment, freshwater or soil environments present a wider variety of physical conditions or habitats. It may be that strains of freshwater *Caulobacters* have holdfasts that attach to specific kinds of surfaces and so may be adapted to more specific niches than is necessary for their marine counterparts.

With current data only some preliminary estimations of holdfast composition can be made. WGA binds to multimers of N-acetylglucosamine, up to a trisaccharide, in preference to a monomer. Thus the binding of WGA to the holdfast and the inability of N-acetylglucosamine to inhibit this binding suggest that the holdfast contains at least short polymers of N-acetylglucosamine. This interpretation is supported by the

observation that the holdfast of a freshwater strain is sensitive to chitinase, since chitin is a polymer of N-acetylglucosamine.

We are in the process of learning what other molecules are constituents of the holdfast and how they are juxtaposed to one another. A complete analysis will likely only be possible after the substance has been isolated in quantity. It seems less likely that the holdfast substance is a glycoprotein; western blot experiments using FITC-WGA as a detection agent have failed to identify protein bands(not shown).

The binding of holdfast to colloidal gold and silver particles is both an interesting and useful finding. Colloidal gold is used as a label for light and electron microscopy and can also be used as a specific stain for blot transfers, due to its intense red color. Thus, as isolation strategies are developed, we have a versatile label in addition to WGA, allowing a variety of assays for holdfast material. The strength of holdfast binding to these particles is an interesting issue. Particles coated with protein A appeared to bind as effectively as those without a protein coating, suggesting that the protein coat is somehow dislodged. Since the binding of proteins to colloidal gold is thought to be very strong(Smit and Todd, 1986) it is possible that there is a true specificity to these heavy metals. A thorough study of the range of surfaces that interact with holdfast material is clearly necessary, but the possibility that this substance has utility in specifically attracting precious and toxic metals is being considered.

The initial work directed to developing or evaluating the capabilities of marine *Caulobacters* for molecular genetic manipulation has been encouraging. For bacteria in which transformation methods have not been developed, the ability to introduce genes by conjugal transfer from *E. coli* is vital. Present information indicates that virtually all the broad host-range plasmids that have been developed for recombinant DNA studies can be used in the marine *Caulobacters*. The ability to readily generate spontaneous mutants would seem a routine finding, but difficulties in obtaining such mutants have been encountered in other marine bacteria(Lidstrom et al, 1984). Additional studies of molecular genetic capabilities are also underway. For example, the observation that the replicon for colicin E1-type plasmids is not recognized in many bacteria other than *E. coli* is the basis for molecular mutagenesis strategies using

conjugal transfer methods(de Bruijn and Lupski, 1984). Whether a similar lack of recognition is true for marine Caulobacters is being tested. We have also discovered native plasmids in several marine Caulobacters and are evaluating their potential for development as stable cloning vectors. The observation that genes from freshwater Caulobacters share homology with the genomes of marine Caulobacters is suggestive that these groups of organisms, although separated by habitat requirements, may be similar genetically as well as morphologically. In turn, this may mean that promoters of cloned freshwater Caulobacter genes are recognized in marine strains, providing a means to develop expression plasmid vectors for the marine strains.

The development of selected marine Caulobacters for molecular genetic experimentation will lead to additional approaches for understanding their participation in biofouling. For example, recombinant DNA methods provide ways to alter the composition of the holdfast in specific ways, using molecular mutagenesis strategies. The effects on adhesiveness or substrate specificity can then be evaluated. A similar approach can be taken for other features, such as surface structures, which may be important for the longterm survival of these fouling bacteria in biofilms.

The availability of cloning tools and strategies for marine Caulobacters also enables using these bacteria for other purposes. We are currently working to engineer the genetic capacity to degrade toxic chemicals, particularly chlorinated hydrocarbons, into marine Caulobacters. In concert with possibilities for genetic manipulations, several of their characteristics, including adhesiveness and longterm survival in nutrient-limited environments(Poindexter, 1981b), make them appear attractive organisms for such work.

ACKNOWLEDGEMENTS

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laboratory, who helped with the conjugation experiments. I also acknowledge the technical assistance of Robert Cranford. This work was supported by grants from the Washington SeaGrant Program, the Office of Naval Research, the National Institutes of Health and the University of California Toxic Substances Research and Teaching Program.

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FIGURE AND TABLE LEGENDS

Fig 1. Negative stain electron microscopy of marine *Caulobacter* MCS 24. The cells are held together by their holdfasts, forming a large rosette. Despite chemical fixation, some membrane damage still occurred when the cells were suspended in distilled water. Bar equals 1 μm .

Fig 2. FITC-WGA labelling. This light micrograph is a combined phase and vertical fluorescence microscopy image which was reproduced from the original color transparency. Thus regions of FITC-WGA label appear as bright areas. A) Rosettes of marine *Caulobacter* MCS 24. The centers of the rosettes are intensely labelled. B) *Caulobacter crescentus* CB2, a freshwater species. The stalk is only barely visible, but the fluorescent label is clearly seen. Bars equal 5 μm .

Fig 3. Effect of WGA on adherence of marine *Caulobacter* MCS 18 cells to glass cover slips. As discussed in the text, all cells seen are firmly attached to the cover slip. A) These cells were treated with *Dolichos biflorus* lectin, which does not bind to the holdfast, prior to the adhesion assay. B) The result of treatment with wheat germ agglutinin, which does bind to the holdfast and effected a significant reduction in attachment.

Fig 4. Binding of colloidal gold and silver particles to *Caulobacter* holdfasts. A) Marine *Caulobacter* MCS 18 labelled with particles of polyethylene glycol-stabilized colloidal gold. B) Freshwater *Caulobacter crescentus* CB2 labelled with protein A-colloidal silver particles. In both cases the labels are specific for the holdfast. Bars equal 0.5 μm .

Table 1. Results of the fluorescent lectin binding assay with marine *Caulobacter* strains. Absence of an "x" indicates a negative result.

Table 2. Results of the fluorescent lectin binding assay with freshwater *Caulobacters*. Strains designated "FWC" are recent isolates.

Table 3. In-progress data on the ability of marine *Caulobacters* to receive plasmids

from E. coli via conjugation. The incompatibility groups listed are those known to have some degree of ability to be transferred between more than one genera of bacteria. pKT230A19 and pKT230E1 are recombinant plasmids containing the gene for the Caulobacter surface protein described in the text. "X" indicates a successful transfer. Absence of an "X" can mean either transfer has not yet been achieved or has not been tried.

LECTIN STRAIN	PEANUT AGGLUTININ	DOLICHOS BIFLORUS	SOYBEAN AGGLUTININ	CONCONAV- ALINA	ULEX EUROPEAUS	WHEAT GERM AGGLUTININ	RICINUS COMMUN
C.CRESCENTUS CB2						+	
C.BACTERIOIDES							
C.LEYDII			+/-	+/-			
C.HENRICI		+				+	
C.VIBROIDES		+/-				+	
C.SUBVIBROIDES		+		+	+		
C.CRESCENTUS CB 1						+	
FWC 2						+	
FWC 4						+	
FWC 7							

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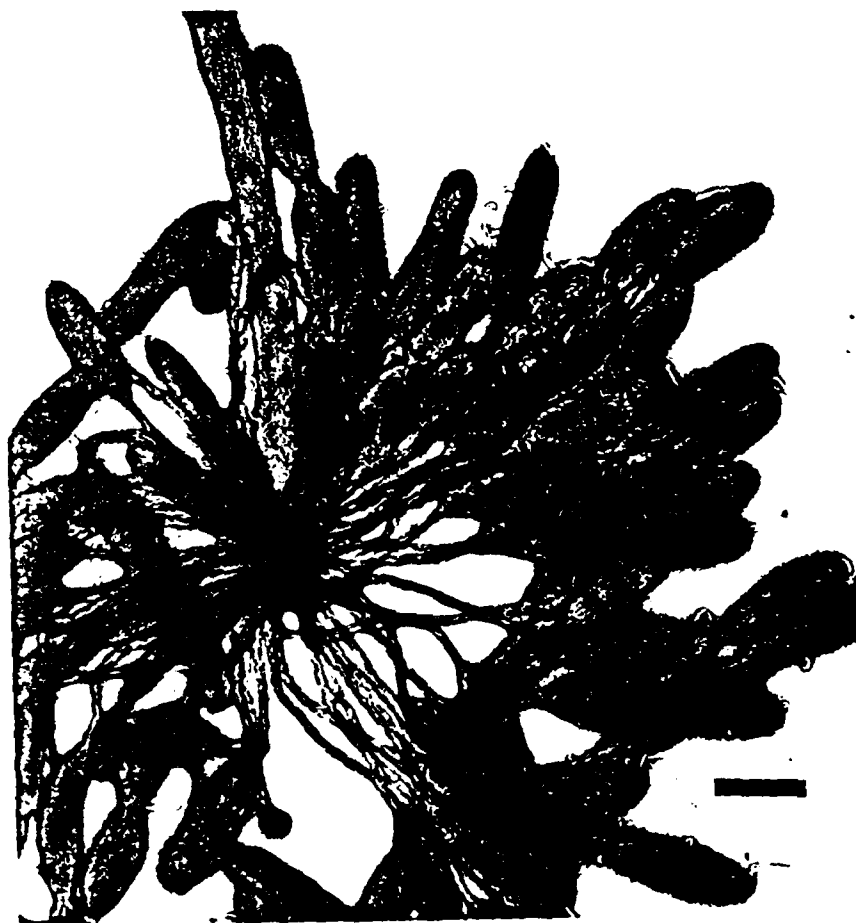
LECTIN STRAIN	PEANUT AGGLUTININ	DOLICHOS BIFLORUS	SOYBEAN AGGLUTININ	CONCONAV- ALINA	ULEX EUROPEAUS	WHEAT GERM AGGLUTININ	RICINUS COMMUNIS
MCS 1						+	
MCS 3						+	
MCS 6						+	
MCS 7						+	
MCS 10						+	
MCS 11						+	
MCS 13						+	
MCS 15						+	
MCS 17						+	
MCS 18						+	
MCS 19						+	
MCS 20						+	
MCS 24						+	
CM 243						+	
CM260							
VC 13						+	

Table 1 *cont.*

MARINE CAULOBACTER STRAINS											Ref.
PLASMIDS	MCS3	MCS5	MCS6	MCS7	MCS9	MCS13	MCS15	MCS17	MCS18	MCS19	
Inc Q R300B pKT230 pGSS33	X	X X	X X X		X		X	X X X	X X X	X	Barth 19 Bagdasarian Shapiro
pKT230A19 pKT230E1	X		X	X		X	X	X X	X X	X	
Inc W pSa151								X			Tait et al 1985
Inc P-1 pRK293			X	X				X			Dittus et al 1985
Inc N R46				X				X	X		Grindley et al 1985

Table 3 Smith

Fish Smit



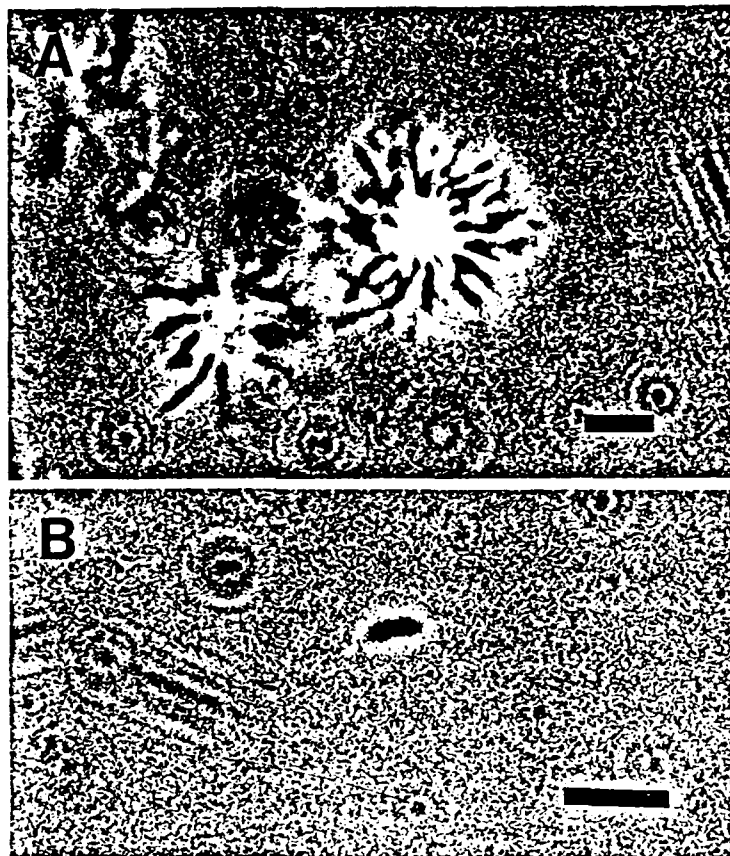


Fig 2 Smif

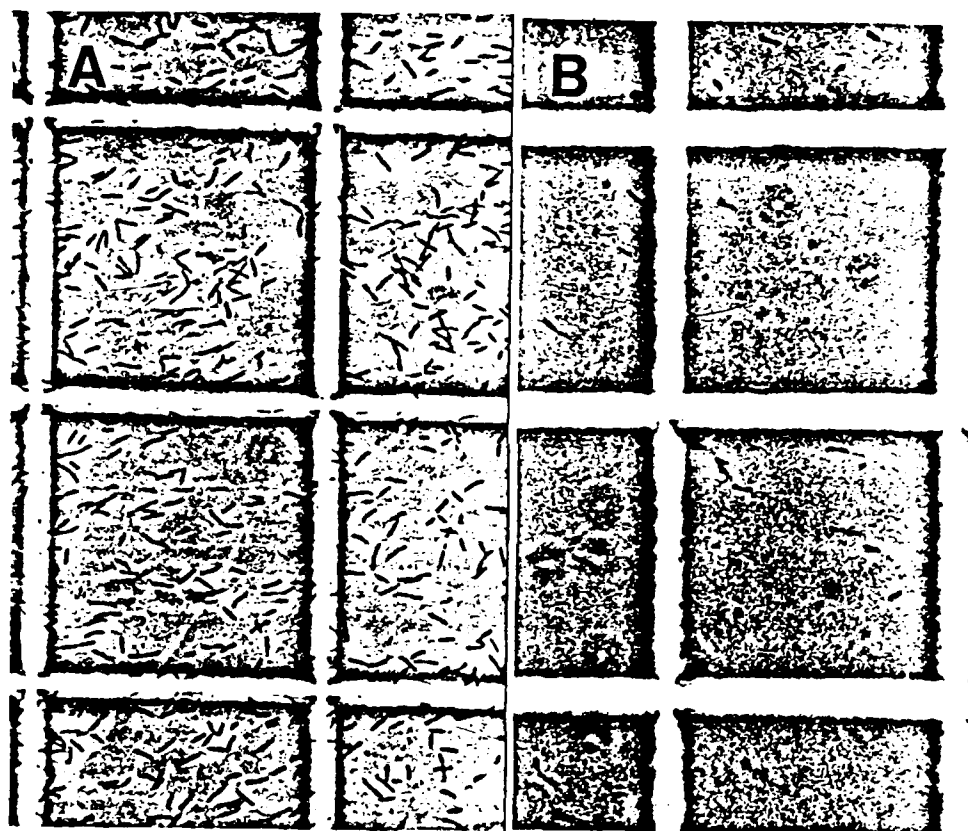


Fig 3 Smit

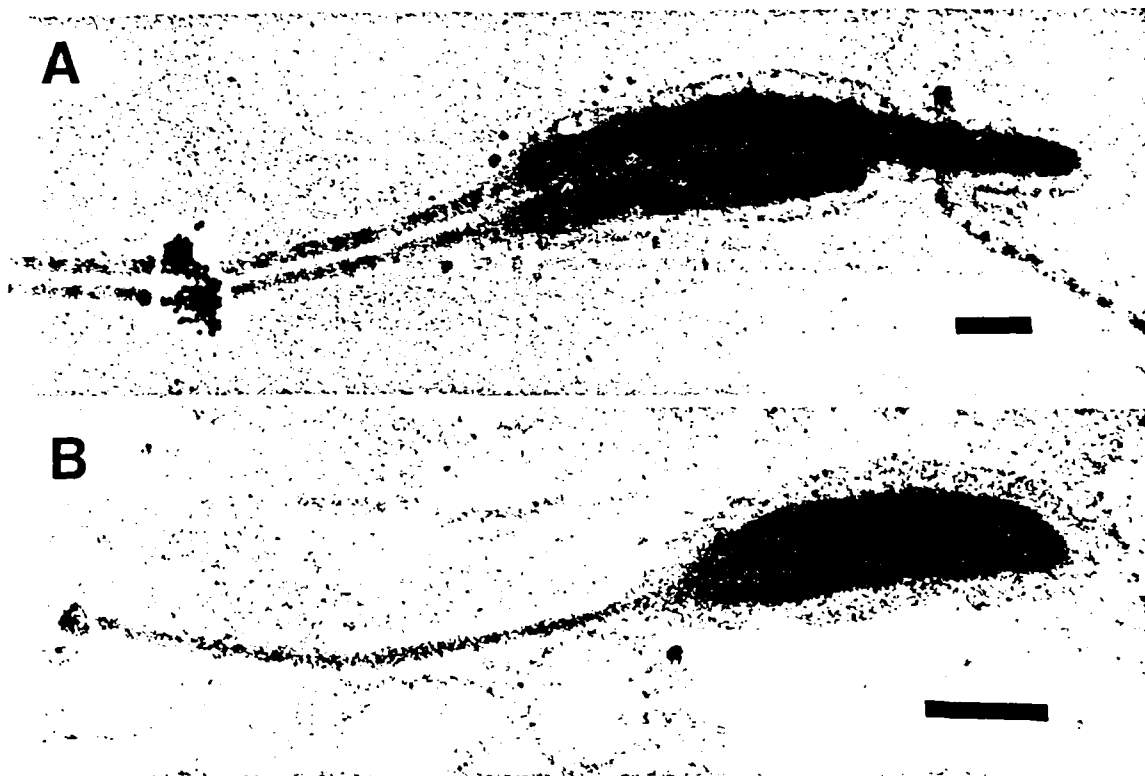


Fig 4 Smif

END

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